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④ Method for the production of erythropoietin.

⑤ The invention refers to recombinant DNA plasmid vector containing cDNA encoding human EPO of clone lambda HEPOFL13 (ATCC 40153) and mammalian cell transformed with this vector or with a plasmid which contains the entire bovine papilloma virus DNA and the cDNA sequences of Table 3 coding for human EPO are which useful in the preparation of recombinant human erythropoietin which is characterized by the presence of O-linked glycosylation.

**METHOD FOR THE PRODUCTION OF ERYTHROPOEITIN**

**FIELD OF THE INVENTION**

The present invention is directed to cloned genes for human erythropoietin that provide surprisingly high expression levels, to the expression of said genes and to the *in vitro* production of active human erythropoietin.

**BACKGROUND OF THE INVENTION**

10 Erythropoietin (hereinafter EPO) is a circulating glycoprotein, which stimulates erythrocyte formation in higher organisms. See, Cannon et al, *Compt. Rend.*, 143:384 (1906). As such, EPO is sometimes referred to as an erythropoietic stimulating factor.

The life of human erythrocytes is about 120 days. Thus, about 1/120 of the total erythrocytes are destroyed daily in the reticulo-endothelial system. Concurrently, a relatively constant number of erythrocytes are produced daily to maintain the level of erythrocytes at all times (Guyton, *Textbook of Medical Physiology*, pp 58-50, W. B. Saunders Co., Philadelphia (1976)).

Erythrocytes are produced by the maturation and differentiation of the erythroblasts in bone marrow, and EPO is a factor which acts on less differentiated cells and induces their differentiation to erythrocytes (Guyton, *supra*).

15 EPO is a promising therapeutic agent for the clinical treatment of anemia or, in particular, renal anemia. Unfortunately, the use of EPO is not yet common in practical therapy due to its low availability. For EPO to be used as a therapeutic agent, consideration should be given to possible antigenicity problems, and it is therefore preferable that EPO be prepared from a raw material of human origin. For example, human blood or urine from patients suffering from aplastic anemia or like diseases who excrete large amounts of EPO may be employed. These raw materials however, are in limited supply. See, for example, White et al, *Rec. Progr. Horm. Res.*, 16:19 (1960); Espada et al, *Blochim. Med.*, 3:475 (1970); Fisher, *Pharmacol. Rev.*, 24:459 (1972) and Gordon, *Vitam. Horm.* 31:105 (1973), the disclosures of which are incorporated herein by reference.

The preparation of EPO products has generally been via the concentration and purification of urine from patients exhibiting high EPO levels, such as those suffering from aplastic anemia and like diseases. See for example, U.S. Patent Nos. 4,397,840, 4,303,650 and 3,885,801 the disclosures of which are incorporated herein by reference. The limited supply of such urine is an obstacle to the practical use of EPO, and thus it is highly desirable to prepare EPO products from the urine of healthy humans. A problem in the use of urine from healthy humans is the low content of EPO therein in comparison with that from anemic patients. In addition, the urine of healthy individuals contains certain inhibiting factors which act against erythropoiesis. In sufficiently high concentration so that a satisfactory therapeutic effect would be obtained from EPO derived therefrom only following significant purification.

20 EPO can also be recovered from sheep blood plasma, and the separation of EPO from such blood plasma has provided satisfactorily potent and stable water-soluble preparations. See, Goldwasser, *Control Cellular Diff. Develop.*, Part A, pp 487-494, Alan R. Liss, Inc., N.Y. (1981), which is incorporated herein by reference. Sheep EPO would however, be expected to be antigenic in humans.

25 Thus, while EPO is a desirable therapeutic agent, conventional isolation and purification techniques, used with natural supply sources, are inadequate for the mass production of this compound.

29 Sugimoto et al, in U.S. Patent No. 4,377,573 describe one method for the mass production of EPO comprising the *in vitro* multiplication of human lymphoblastoid cells, including Namalwa, BALL-1, NALL-1 and JBL.

The reported production by others of EPO using genetic engineering techniques had appeared in the trade literature. However, neither an enabling disclosure nor the chemical nature of the product has yet been published. In contrast, the present application provides an enabling disclosure for the mass production of proteins displaying the biological properties of proteins displaying the biological properties of human EPO. It is also possible by such techniques to produce proteins which may chemically differ from authentic human EPO, yet manifest similar (and in some cases improved) properties. For convenience all such proteins displaying the biological properties of human EPO may be referred to hereinafter as EPO whether or not chemically identical thereto.

SUMMARY OF THE INVENTION

The present invention is directed to the cloning of a gene that expresses surprisingly high levels of human EPO, the expression thereof, and the mass production *in vitro* of active human EPO. Expression vectors, plasmid and related processes. Described also are suitable expression vectors for the production of EPO, expression cells, purification schemes and related processes.

As described in greater detail infra, EPO was obtained in partially purified form and was further purified to homogeneity and digested with trypsin to generate specific fragments. These fragments were purified and sequenced. EPO oligonucleotides were designed based on these sequences and synthesized. These oligos were used to screen a human genomic library from which was isolated an EPO gene.

The EPO gene was verified on the basis of its DNA sequence which matched many of the hypoxic protein fragments sequenced. A piece of the genomic clone was then used to demonstrate that EPO mRNA could be detected in human fetal (20 week old) mRNA. A human fetal liver cDNA library was prepared and screened. Three EPO cDNA clones were obtained (after screening >750,000 recombinants). Two of these clones were determined to be full length as judged by complete coding sequence and substantial 5-prime and 3-prime untranslated sequence. These cDNAs have been expressed in both SV-40 virus transformed monkey cells (the COS-1 cell line; Gluzman, Cell 23:175-182 (1981)) and Chinese hamster ovary cells (the CHO cell line; Urbach, G. and Chasin, L. A. Proc. Natl. Acad. Sci. USA 77:4216-4220 (1980)). The EPO produced from COS cells is biologically active EPO *in vitro* and *in vivo*. The EPO produced from CHO cells is also biologically active *in vitro* and *in vivo*.

The EPO cDNA clone has an interesting open reading frame of 14-15 amino acids (aa) with initiator and terminator from 20 to 30 nucleotides (nt) upstream of the coding region. A representative sample of E. coli transfected with the cloned EPO gene has been deposited with the American Type Culture Collection, Rockville, Maryland, where it is available under Accession Number ATCC 40153.

BRIEF DESCRIPTION OF DRAWINGS AND TABLES

Table 1 is the base sequence of an 87 base pair exon of a human EPO gene;

Figure 1 illustrates the detection of EPO mRNA in human fetal liver mRNA;

Table 2 illustrates the amino acid sequence of an EPO protein deduced from the nucleotide sequence of lambda-HEPOFL13;

Table 3 illustrates the nucleotide sequence of the EPO cDNA in lambda-HEPOFL13 (shown schematically in Figure 2) and the amino acid sequence deduced therefrom;

Figure 3 illustrates the relative positions of DNA inserts of four independent human EPO genomic clones;

Figure 4 illustrates a map of the apparent human and exon structure of the human EPO gene;

Table 4 illustrates a DNA sequence of the EPO gene illustrated in Figure 4;

Figures 5A, 5B and 5C illustrate the construction of the vector 91023(B);

Figure 6 illustrates SDS polyacrylamide gel analysis of EPO produced in COS-1 cells compared with native EPO;

Table 5 illustrates the nucleotide and amino acid sequence of the EPO clones, lambda-HEPOFL6;

Table 6 illustrates the nucleotide and amino acid sequence of the EPO clones, lambda-HEPOFL8;

Table 7 illustrates the nucleotide and amino acid sequence of the EPO clone lambda-HEPOFL13;

Figure 7 is a schematic illustration of the plasmid pRK14; and

Figure 8 is a schematic illustration of the plasmid pDBPV-MMTneo(342-12).

DETAILED DESCRIPTION

The present invention is directed to the cloning of EPO genes and to the production of EPO by the *in vitro* expression of those genes.

The patent and scientific literature is replete with processes reportedly useful for the production of recombinant products. Generally, these techniques involve the isolation or synthesis of a desired gene sequence, and the expression of that sequence in either a prokaryotic or eukaryotic cell, using techniques commonly available to the skilled artisan. Once a given gene has been isolated, purified and inserted into a transfer vector (i.e., cloned), its availability in substantial quantity is assured. The vector with its cloned gene is transferred to a suitable micro-organism or cell line, for example, bacteria, yeast, mammalian cells such

as, COS-1 (monkey kidney), CHO (Chinese hamster ovary), insect cell lines, and the like, wherein the vector replicates as the microorganism or cell line proliferates and from which the vector can be isolated by conventional means. Thus there is provided a continuously renewable source of the gene for further manipulations, modifications and transfers to other vectors or other loci within the same vector.

Expression may often be obtained by transferring the cloned gene, in proper orientation and reading frame, into an appropriate site in a transfer vector such that translational read-through from a prokaryotic or eukaryotic gene results in synthesis of a protein precursor comprising the amino acid sequence coded by the cloned gene linked to Met or an amino-terminal sequences to the prokaryotic or eukaryotic gene. In other cases, the signals for transcription and translation initiation can be supplied by a suitable genomic fragment of the cloned gene. A variety of specific protein cleavage techniques may be used to cleave the protein precursor, if produced, at a desired point so as to release the desired amino acid sequence, which may then be purified by conventional means. In some cases, the protein containing the desired amino acid sequence is produced without the need for specific cleavage techniques and may also be released from the cells into the extracellular growth medium.

Isolation of a Genomic Clone of Human EPO

Human EPO was purified to homogeneity from the urine of patients afflicted with aplastic anemia as described infra. Complete digestion of this purified EPO with the protease trypsin, yielded fragments which were separated by reverse phase high performance liquid chromatography, recovered from gradient fractions, and subjected to micro-sequence analysis. The sequences of the tryptic fragments are undefined in Tables 2 and 3 and are discussed in more detail infra. Two of the amino acid sequences, Val-Asn- $\text{Phi}$ -Tyr-Ala-Trp-Lys and Val-Tyr-Sar-Asn-Phe-Lys-Arg, were chosen for the design of oligonucleotide probes (resulting in an oligonucleotide pool 17nt long and 32-fold degenerate, and an oligonucleotide pool 18nt long and 128-fold degenerate, from the latter tryptic fragment, as well as two pools 14nt long, each 48-fold degenerate, from the latter tryptic fragment, respectively). The 32-fold degenerate 17mer pool was used to screen a human genomic DNA library in a *Ch4A* vector (22) using a modification of the Woo and O'Malley *in situ* amplification procedure (47) to prepare the filters for screening.

As used herein, arabic numbers in parentheses, (1) through (81), are used to refer to publications that are listed in numerical order at the end of this specification. Phage hybridizing to the 17mer were plated, pooled in small groups and probed with the 14mer and 18mer pools. Phage hybridizing to the 17mer were plated, pooled in small groups and probed with the 14mer and 18mer pools. Phage hybridizing to the 17mer, 18mer and 14mer pools were plaque purified and fragments were subcloned into M13 vectors for sequencing by the dideoxy chain termination method of

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atttGGatGAAAGGGaaGaaGatG

ACG GGC TGT GCT GAA CAC TGC AGC TGA AAT GAG AAT ATC  
Thr Gly Cys Ala Gln Ile Cys Ser Leu Asn Gln Asn Ile  
ACT GTC CCA GAC ACC AAA GTC ATT TTC TAT GCC Trp Lys  
Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Ala  
ACG ATG GAGTGGatGAAAGGGaaGaaGatG

TABLE 1

atttGGatGAAAGGGaaGaaGatG

ACG GGC TGT GCT GAA CAC TGC AGC TGA AAT GAG AAT ATC  
Thr Gly Cys Ala Gln Ile Cys Ser Leu Asn Gln Asn Ile  
ACT GTC CCA GAC ACC AAA GTC ATT TTC TAT GCC Trp Lys  
Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Ala  
ACG ATG GAGTGGatGAAAGGGaaGaaGatG

ALA TRP LEU TRP LCU LCU LEU SER LEU LEU PRO GLY LEU PRO VAL LEU GLY  
-27 HET GLY VAL HIS GLU CYS PRO  
ALA Pro Pro ARG Ile Cys Asp Ser Val Arg Tyr Leu Leu Gln  
-20 10 20 30 40 50 60  
Glu Ala Gln -Asn Ile Thr -Thr -Gly Cys Ala Gln Ile Ser Leu Asn Gln Asn Ile Thr  
Glu Ala Gln -Asn Ile Thr -Thr -Gly Cys Ala Gln Ile Ser Leu Asn Gln Asn Ile Thr  
Val Pro Asp Thr Lys Val Asn Phe Tyr Lys Arg Met Gln Gln Gln  
60 50 40 30 20 10

TABLE 2

ALA Pro Pro Arg Ile Cys Asp Ser Val Arg Tyr Leu Leu Gln  
-20 10 20 30 40 50 60  
Glu Ala Gln -Asn Ile Thr -Thr -Gly Cys Ala Gln Ile Ser Leu Asn Gln Asn Ile Thr  
Glu Ala Gln -Asn Ile Thr -Thr -Gly Cys Ala Gln Ile Ser Leu Asn Gln Asn Ile Thr  
Val Pro Asp Thr Lys Val Asn Phe Tyr Lys Arg Met Gln Gln Gln  
60 50 40 30 20 10

6 10 15 20 25 30 35 40 45 50

EPO genomic clones has been obtained by sequencing additional exons containing other typical fragment coding information.

Isolation of EPO cDNA Clones

**Northern Analysis** (55) of human fetal (20 week old) liver mRNA was conducted using a 65nt single-stranded probe prepared from an M13 clone containing a portion of the 87P exon described in Table 1. As illustrated in Figure 1, a strong signal could be detected in fetal liver mRNA. The precise identification of this band as P40 mRNA was achieved by using the same probe to screen a bacteriophage lambda cDNA library of the fetal liver mRNA (25). Several hybridizing clones were obtained at a frequency of approximately 1 positive per 250,000 recombinants screened. The complete nucleotide and deduced amino acid sequences for these clones (lambda-HEPOFL13 and lambda-HEPOFL4) are shown in Tables 5 and 6. The EPO coding information is contained within 594nt in the 5-prime half of the cDNA, including a very long poly-A tail and a 400 nucleotide poly-A signal motif.

hypothetical  $\alpha_2$  amino acid residue and the two amino acids in mature protein.

The identification of the N-terminus of the mature protein was based on the N-terminal sequences of the protein secreted in the urine of persons with aplastic anemia as illustrated herein (Table 1), and as published by Goldwasser (26), Sue and Strykowski (27), and by Yangawa (21). Whether this N-terminus (Ala-Pro-Pro-Arg $\rightarrow$ ) represents the actual N-terminus found on EPO in circulation or whether some cleavage occurs in the kidney or urine is presently unknown.

The amino acid sequences which are underlined in Tables 2 and 3 indicate those tryptic fragments or the portion of the N-terminus for which protein sequence information was obtained. The deduced amino acid sequence agrees precisely with the tryptic fragments which have been sequenced, confirming that the

Structure and Sequence of the Human EBO Gene

The relative positions of the DNA inserts of four independent human EPO genomic clones are shown in Figure 3. Hybridization analysis of these cloned DNAs with oligonucleotide probes and with various probes prepared from the two classes of EPO cDNA clones positioned the EPO gene within the approximately 3.3 kb region shown by the darkened line in Figure 3. Complete sequence analysis of this region (see Example 4) and comparison with the cDNA clones, resulted in the map of the intron and exon structure of the EPO gene shown in Figure 4. The EPO gene is divided into 5 exons. Part of exon I, all of exons II, III and IV, and part of exon V, contain the protein coding information. The remainder of exons I and V encodes the 5'prime and 3'prime untranslated portions, respectively.

## Transient Expression of EPO In COS Cells

To demonstrate that biologically active EPO could be expressed in an *in vitro* cell culture system, COS cell expression studies were conducted (53). The vector used for the transient transfection, pGK023(B), is described in Example 5. This vector contains the adenovirus major late promoter, an SV40 polyadenylation sequence, an SV40 origin of replication, SV40 enhancer, and the adenovirus VA gene. The cDNA insert in lambda-HEPOFL13 (see Table 6) was inserted into the pGK023(B) vector, downstream of the adenovirus

Twenty four hours after transfection of this construct into the NIH 3T3 cells (Herowitz et al. J. Mol. Appl. Genet. 2:147-149 (1983)) the cells were washed, changed to serum free media, and the cells were harvested 48 hrs. later. The level of release of EPO into the culture supernatant was then examined using a quantitative radioimmunoassay for EPO (55). As shown in Table 8, (Example 6) Immunologically reactive EPO was expressed. The biological activity of the EPO produced from COS-1 cells was examined. In a separate experiment, the vector containing EPO cDNA from lambda-HEPO13 was transfected into COS-1 cells and media harvested as described supra. EPO in the media was then quantified by the either of two *in vitro* biological assays,  $^{3}\text{H}$ -thymidine and CFU-E (12, 28), and by either of two *in vivo* assays, hypoxic mouse and starved rat (30, 31) (see Table 8, Example 7). These results demonstrate that biologically active EPO is produced in COS-1 cells. By Western blotting, using a polyclonal anti-EPO antibody, the EPO produced by COS cells has a mobility on SDS-polyacrylamide gels which is identical to that of native EPO measured from human urine (Example 8). Thus, the extent of major late promoter. This new vector is identified as pPT1L3.

Sanger and Coulson, (23) (1977). The sequence of the region hybridizing to the 32-fold degenerate 17mer in one of the clones is shown in Table 1. This DNA sequence contains within an open reading frame, the nucleotides which could precisely code for the tryptic fragment used to deduce the 17mer pool of oligonucleotides. Furthermore, analysis of the DNA sequence indicated that the 17mer hybridizing region was contained within an 87bp exon, bounded by potential splice acceptor and donor sites.

Different vectors containing other promoters can also be used in COS cells or in other mammalian or eukaryotic cells. Examples of such other promoters useful in the practice of this invention include SV40 early and late promoters, the mouse metallothionein gene promoter, the promoter found in the long terminal repeats of avian or mammalian retroviruses, the bacteriophage polyhedrin gene promoter and others. Examples of other cell types useful in the practice of this invention include E. coli, yeast, mammalian cells such as CHO (Chinese hamster ovary), C127 (monkey epithelium), 3T3 (mouse fibroblast) CV-1 (African green monkey kidney), and the insect cells such as those from *Spodoptera frugiperda* and *Drosophila melanogaster*. These alternate promoters and/or cell types may enable regulation of the timing or level of EPO expression, producing a cell-specific type of EPO, or the growth of large quantities of EPO producing cells under less expensive, more easily controlled conditions.

An expression system which retains the benefits of mammalian expression but requires less time to produce a high-level expression cell line is composed of an insect cell line and a DNA virus which reproduces in this cell line. The virus is a nuclear polyhedrosis virus. It has a double-stranded circular DNA genome of 128 kb. The nucleocapsid is rod-shaped and found packaged in two forms, the non-occluded form, a membrane budded virus and an occluded form, packaged in a protein crystal in the infected cell nucleus. These viruses can be routinely propagated in *in vitro* insect cell culture and are amenable to all routine animal virological methods. The cell culture media is typically a nutrient salt solution and 10% fetal calf serum.

*In vitro* virus growth is initiated when a non-occluded virus (NOV) enter a cell and moves to the nucleus where it replicates. Replication is nuclear. During the initial phase (8-18 hrs. post-infection) of viral application, nucleocapsids are assembled in the nucleus and subsequently bud through the plasma membrane as NOVs, spreading the infection through the cell culture. In addition, some of the nucleocapsids subsequently (18+ hrs. post-infection) remain in the nucleus and are occluded in a protein matrix, known as the polyhedral inclusion body (PIB). This form is not infectious in cell culture. The matrix is composed of a protein known as polyhedrin, MW 33 kD. Each PIB is approximately 1 mm in diameter, and there can be as many as 100 PIBs per nucleus. There is clearly a great deal of polyhedrin produced late in the infection cycle, as much as 25% of total cellular protein.

Because the PIB plays no role in the *in vitro* replication cycle, the polyhedrin gene can be deleted from the virus chromosome with no effect on *In Vitro* viability. In using the virus as an expression vector, we have replaced the polyhedrin gene coding region with the foreign DNA to be expressed, placing it under the control of the polyhedrin promoter. This results in a non-PIB forming virus phenotype.

This system has been utilized by several researchers the most noted being Pennoch et al. and Smith et al. Pennoch et al. (Gregory D. Pennoch, Charles Shoemaker, and Lois K. Miller, Molecular and Cell Biology 3:84, p. 399-406) have reported on the high level expression of a bacterial protein,  $\beta$ -galactosidase, which placed under the control of the polyhedrin promoter.

Another nuclear polyhedrosis virus-derived expression vector has been presented by Smith et al. (Gale E. Smith, Max D. Summers and M. J. Fraser, Molecular and Cell Biology, May 16, 1983, pp. 2158-2165). They have demonstrated the effectiveness of their vector through the expression of human B-interferon. The synthesized product was found to be glycosylated and secreted from insect cells, as would be expected. In Example 14, modifications to the plasmid containing the Autographa californica nuclear polyhedrosis virus (AcNPV) polyhedron gene are described which allow the easy insertion of the EPO gene into the plasmid so that it may be under the transcriptional control of the polyhedrin promoter. The resulting DNA is transfected with intact chromosomal DNA from wild type AcNPV into insect cells. A genetic recombination event results in the replacement of the AcNPV/C polyhedrin gene region with the DNA from the plasmid. The resulting recombinant virus can be identified amongst the viral progeny by its possession of the DNA sequences of the EPO gene. This recombinant virus, upon reinfection of insect cells is expected to produce EPO.

Examples of EPO expression in CHO, C127 and 3T3, and insect cells are given in Examples 10 and 11

(CHO), 13 (C127 and 3T3) and 14 (insect cells). Recombinant EPO produced in CHO cells as in Example 11 was purified by conventional column chromatographic methods. The relative amounts of sugars present in the glycoprotein were analyzed by two independent methods [(i) Reinhold, Methods in Enzymol. 50:244-249 (Methanols) and (ii) Takemoto, H. et al., Anal. Biochem. 145:245 (1985) (aspartyl amination together with independent steric acid determination)]. The results obtained by each of these methods were in excellent agreement. Several determinations were thus made, yielding the following average values wherein N-acetylglucosamine is, for comparative purposes, given a value of 1:

	Sugar	Relative molar level
6	N-Acetylglucosamine	1
	Hexasaccharide	1.4
	Galactose	0.9
	Mannose	0.5
	N-Acetylneurameric acid	1
	Fucose	0.2
10	N-Acetylglucosamine	0.1

It is noteworthy that significant levels of fucose and N-acetylglucosamine were reproducibly observed using both independent methods of sugar analysis. The presence of N-acetylglucosamine indicates the presence of O-linked glycosylation on the protein. The presence of O-linked glycosylation was further indicated by SDS-PAGE analysis of the glycoprotein following digestion of the glycoprotein with various combinations of glycosidic enzymes. In particular, following enzymatic removal of all N-linked carbohydrate on the glycoproteins using the enzyme peptide endo F, N-glycosidase, the molecular weight of the protein was further reduced upon subsequent digestion with neuramidase, as determined by SDS-PAGE analysis.

15 *In vitro* biological activity of the purified recombinant EPO was assayed by the method of G. Krystal, Exp. Hematol. 11:149 (1983) ( spleen cell proliferation bioassay) with protein determinations calculated based upon amino acid composition data. Upon multiple determinations, the *In vitro* specific activity of the purified recombinant EPO was calculated to be greater than 200,000 units/mg protein. The average value was in the range of about 275,000 - 300,000 units/mg protein. Moreover, values higher than 300,000 have also been observed. The *In vitro* (polyclonal mouse assay, Kazal and Erslev, Am. Clin. Lab. Sci., Vol. 3, p. 91 (1975)) *In vitro* activity ratios observed for the recombinant material was in the range of 0.7 - 1.3.

20 It is interesting to compare the glycoprotein characterization presented above with the characterization for a recombinant CHO-produced EPO material previously reported in International Patent Application Publication No. WO 8502610 (published 20 June 1985). The corresponding comparative sugar analysis described on page 65 of that application a value of zero for fucose and for N-acetylglucosamine and a hexose:N-acetylglucosamine ratio of 15.0:1. The absence of N-acetylglucosamine indicates the absence of O-linked glycosylation in the previously reported glycoprotein. In contrast to that material, the recombinant CHO-produced EPO of this invention which is characterized above contains significant and reproducible observable amounts of both fucose and N-acetylglucosamine, contains less than one-tenth, 25 the relative amount of hexoses and is characterized by the presence of O-linked glycosylation. Furthermore, the high specific activity of the above-described CHO-derived recombinant EPO of this invention may be directly related to its characteristic glycosylation pattern.

The biologically active EPO produced by the procaryotic or eukaryotic expression of the cloned EPO genes of the present invention can be used for the *In vivo* treatment of mammalian species by physicians and/or veterinarians. The amount of active ingredient will, of course, depend upon the severity of the condition being treated, the route of administration chosen, and the specific activity of the active EPO, and ultimately will be decided by the attending physician or veterinarian. Such amount of active EPO was determined by the attending physician is also referred to herein as an "EPO treatment effective" amount. 30 For example, in the treatment of induced hypotrophic anemia associated with chronic renal failure in sheep, an effective daily amount of EPO was found to be 10 units/kg for from 15 to 40 days. See Eschbach et al., J. Clin. Invest., 74:34 (1984).

35 The active EPO may be administered by any route appropriate to the condition being treated. Preferably, the EPO is injected into the bloodstream of the mammal being treated. It will be readily appreciated by those skilled in the art that the preferred route will vary with the condition being treated.

40 While it is possible for the active EPO to be administered as the pure or substantially pure compound, it is preferable to present it as a pharmaceutical formulation or preparation. The formulations of the present invention, both for veterinary and for human use, comprise an active EPO protein, as above described, together with one or more pharmaceutically acceptable carriers and/or 45 optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. Desirably the formulation should not include oxidizing agents and other substances with which peptides are known to be incompatible. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of

bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired formulation.

(New England Nuclear and incubated overnight at 37 deg. on fresh NZCYM plates. The filters were then denatured and neutralized by floating for a 10 min. each on a thin film of 0.5N NaOH - 1M NaCl and 0.3M Tris (pH 8) - 1M NaCl respectively. Following vacuum baking at 80 deg. for 2 hrs., the filters were washed in 5% SSC, 0.5% SDS for 1 hr. and the cellular debris on the filter surface was removed by gentle scraping with a wet tissue. This scraping reduced the background binding of the probe to the filters. The filters were then rinsed with  $H_2O$  and presoaked for from 4 to 8 hrs. at 48 deg. in 3M tetramethylammonium

formulations may be conveniently prepared by dissolving some active ingredient in water to produce an aqueous solution, and rendering said solution sterile may be presented in unit or multi-dose containers, for

EPOcDNA as used herein includes the mature EPOcDNA gene preceded by an ATG codon and EPOcDNA coding for allelic variations of EPO protein. One allele is illustrated in Tables 2 and 3. The example sealed ampoules or vials.

The following examples are provided to aid in the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth, without departing from the spirit of the invention. All temperatures are expressed in degrees Celsius and are uncorrected. The symbol for micron or micra, e.g. microliter, micromole, etc., "μ" is an "μ" with a dot over it.

EXAMPLES

## Example 1: Isolation of a Gamma Class of EBO

EPO was purified from the urine of patients with aplastic anemia essentially as described previously (Miyake, et al., *J. Biol. Chem.*, **252**:5558 (1977)) except that the phenol treatment was eliminated and replaced by heat treatment at 80 deg. for 5 min. to inactivate neuraminidase. The final step in the purification was fractionation on a C-4 Yeda HPLC column (The Separations Group) using 0 to 95% acetonitrile gradient with 0.1% trifluoroacetic acid (TFA) over 100 minutes. The position of EPO in the acetonitrile gradient was determined by gel electrophoresis and N-terminal sequence analysis (21, 26, 27) of the major gradient was monitored by gel electrophoresis and N-terminal sequence analysis (21, 26, 27) of the major peaks. The EPO was eluted at approximately 53% acetonitrile and represented approximately 40% of the protein subjected to reverse phase - HPLC. Fractions containing EPO were evaporated to 60  $\mu$ l, adjusted to pH 7.0 with ammonium bicarbonate digested to completion with 2% TPCK-treated trypsin (Worthington) for 18 hrs. at 37 deg. The tryptic digestion was then subjected to reverse phase HPLC as described above. The optical density at both 280 and 214 nm was monitored. Well separated peaks were evaporated to dryness, and subjected directly to N-terminal amino acid sequence analysis (59) using an Applied Biosystems model 480A gas phase sequenator. The sequences obtained are underlined in Tables 2 and 3. As described herein supra, two of these tryptic fragments were chosen for synthesis of oligonucleotide

(New England Nuclear and incubated overnight at 37 deg. on fresh NZCYM plates. The filters were then denatured and neutralized by floating for a 10 min. each on a thin film of 0.5N NaOH • 1M NaCl and 0.5M Tris (pH 6) • 1M NaCl respectively. Following vacuum baking at 80 deg. for 2 hrs., the filters were washed in 5 x SSC, 0.5% SDS for 1 hr. and the cellular debris on the filter surface was removed by gentle scraping with a wet tissue. This scraping reduced the background binding of the probe to the filters. The filters were then rinsed with H<sub>2</sub>O and prehybridized for from 4 to 8 hrs. at 48 deg. In 3M tetramethylammonium chloride, 10 mM NaPO<sub>4</sub> (pH 6.8), 6 x Denhardt's, 0.5% SDS and 10 mM EDTA. The <sup>32</sup>P-labeled 17mer was then added at a concentration of 0.1 pmol/ml and hybridization was carried out at 48 deg. for 72 hrs. Following hybridization the filters were washed extensively in 2 x SSC (0.3M NaCl • 0.03M Na citrate, pH 7) at a room temperature and then rinsed with 1 hr. in 3M TMAIC • 10 mM NaPO<sub>4</sub> (pH 6.8) at room temperature and from 5 to 15 min. at the hybridization temperature. Approximately 120 strong duplicate signals were detected following 2 day autoradiography with an intensifying screen. The positives were picked, grouped in pools of 8, replicated and rescreened in triplicate using one-half of the 14mer pool on each of two filters and the 127mer on the third filter. The conditions for picking and hybridization were as described supra except that hybridization for the 14mer was at 37 deg. Following autoradiography, the probe was removed from the 17mer filter in 50% formamide for 20 min. at room temperature and the filter was rehybridized at 52 deg. with the 18mer probe. Two independent phage hybridized to all three probes. DNA from one of these phage (designated herein, lambda HEPO1) was digested to completion with Sau3A and subcloned into M13 for DNA sequence analysis using the dideoxy chain termination method of Sanger and Coulson, (23) (1977). The nucleotide sequence and deduced amino acid sequence of the open reading frame coding for the HEPO tryptic fragment (underlined region) are described herein. Intron sequences are given in lower case letters; exon sequences (87 nt) are in upper case. Sequences which agree with consensus splice acceptor (a) and donor (d) sites are underlined. (See Table 4.)

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5  $\mu$ g of human fetal liver mRNA (prepared from a 20 week old fetal liver) and adult liver mRNA were electro

(Miyake, et al., 1991) except that the protein sequences were determined on a C-4 Vydac HPLC column (The Separations Group) using 0 to 85% acetonitrile gradient with 0.1% trifluoroacetic acid (TFA) over 100 minutes. The position of EPO in the gradient was determined by gel electrophoresis and N-terminal sequence analysis (21, 26, 27) of the peaks. The EPO was eluted at approximately 55% acetonitrile and represented approximately 40% of the protein subjected to reverse phase - HPLC. Fractions containing EPO were evaporated to 100  $\mu$ l, adjusted to pH 7.0 with ammonium bicarbonate digested to completion with 2% TPCK-treated trypsin (Worthington) for 18 hrs. at 37 deg. The tryptic digestion was then subjected to reverse phase HPLC as described above. The critical density at both 280 and 214 nm was monitored. Well separated peaks were evaporated to near dryness, and subjected directly to N-terminal amino acid sequence analysis (59) using an Applied Biosystems Model 460A gas phase sequenator. The sequences obtained are underlined in Tables 2 and 3. As described herein supra, two of these tryptic fragments were chosen for syntheses of oligonucleotide

TABLE 4 (CONT.)

5 10 15 20 25 30 35 40 45 50 55

TABLE 4

screening (Benton Davis, Sciences, [54] (1978) procedures. Three independent positive clones (designated hereafter, lambda-HEPOFL6 (7350bp), lambda-HEPOFL9 (700bp) and lambda-HEPOFL13 (1400bp) were isolated following screening of  $1 \times 10^6$  plaques. The entire insert of lambda-HEPOFL13 and lambda-HEPOFL9 were sequenced following subcloning into M13 (Tables 7 and 5, respectively). Only portions of lambda-HEPOFL6 was sequenced and the remainder assumed to be identical to the other two clones. (Table 6). The 5-prime and 3-prime untranslated sequences are represented by lower case letters. The coding region is represented by upper case letters.

TABLE 4 (CONT.)

45 phoresed in a 0.8% agarose formaldehyde gel and transferred to nitrocellulose using the method of Berman et al. Cell 23:731 (1981). A single-stranded probe was then prepared from an M13 template containing the 46 insert illustrated in Table 1. The primer was a 20mer derived from the same tryptic fragment as the original 17mer probe. The probe was prepared as previously described by Arnsdorf et al., PNAS, 81 (1984) except that, following digestion with Smal (which produced the desired probe of 85nt length containing 74nt of coding sequences), the small fragment was purified from the M13 template by chromatography on a 50 sepharose C14B column in 0.1M NaOH - 0.2M NaCl. The filter was hybridized to approximately 5 x 10<sup>5</sup> cpm of this probe for 12 hrs. at 68 deg., washed in 2 x SSC at 88 deg., and exposed for 6 days with an intensifying screen. A single marker mRNA of 1200 nt (indicated by the arrow) was run in an adjacent lane.

A probe identical to that described in Example 2 was prepared and used to screen a fetal liver cDNA library prepared in the vector lambda-Ch21A (Todle et al., *Nature*, 25 (1984)) using standard plaque

TABLE 5 (CONT.)

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TABLE 6 (CONT.)

40 45 50 55

sequence of certain trypic fragments which could not be determined unambiguously. The cDNA clones lambda-HEPOFL8, lambda-HEPOFL8 and lambda-HEPOFL13 have been deposited and are available from the American Type Culture Collection, Rockville, Maryland as Accession Numbers ATCC 40156, ATCC 40152 and ATCC 41753, respectively.

#### Example 4: Genomic Structure of the EPO Gene

The relative sizes and positions of four independent genomic clones (lambda-HEP01, 2, 3, and 6) from the Haell/Alu library are illustrated by the overlapping lines in Figure 3. The thickened line indicates the position of the EPO gene. A scale in kb and the positions of known restriction endonuclease cleavage sites are shown. The region containing the EPO gene was completely sequenced from both strands using directed exonucleases. The generated series of deletions through this region. A schematic representation of five exons coding for EPO mRNAs is shown in Figure 4. The precise 5'-prime boundary of exon 1 is presently unknown. The protein coding portion of the exons are indicated. The complete nucleotide sequence of the region is shown in Table 4. The known limits of each exon are delineated by the solid vertical bars. Genomic clones lambda-HEP01, lambda-HEP02, lambda-HEP03 and lambda-HEP06 have been deposited and are available from the American Type Culture Collection, Rockville, Maryland as Accession Numbers ATCC 40154, ATCC 40155, ATCC 40150, and ATCC 40151, respectively.

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### Example 5: Construction of Vector B91023(b)

The transformation vector was pAD258VpA(3) described by Kaufman et al., *Mol. Cell Biol.* 21:1304 (2001). Briefly, this plasmid contains a mouse fibroblast-like cell line (DFHR) cDNA gene that is under transcriptional control of the adenovirus 2 (Ad2) major late promoter. A 5-prime splice site is indicated in the adenovirus DNA and a 3-prime splice site, derived from the immunoglobulin gene, is present between the Ad2 major late promoter and the DFHR coding sequence. The SV40 early polyadenylation site is present downstream from the DFHR coding sequence. The pAD258VpA(3) is from pSYCD (Mellon et al., *Cell*, 27: 278 (1981)) and does not contain the pRS22 sequences known to inhibit replication in mammalian cells (Lusky et al., *Nature*, 332: 79 (1988)).

prime sites of the  $\lambda$ -loop insertion. This plasmid is designated pBR322.

The Ava II D fragment of SV40 containing the SV40 enhancer sequence was obtained by digesting SV40 DNA with Ava II, blunting the ends with the Klenow fragment of  $\lambda$  DNA polymerase, ligating Xba I linkers to the fragments, digesting with Xba I to open the Xba I and isolating the fourth largest (D) fragment by gel electrophoresis. This fragment was then ligated to Xba I cut pT<sub>PL</sub>, yielding the plasmid pCYSV12-TPL. The orientation of the SV40 D fragment in pCYSV12-TPL was such that the SV40 late promoter was in the same orientation as the adenovirus major late promoter.

To introduce the adenovirus (A) genes into the pCYSV12-TPL, first a plasmid pBR322 was constructed that contained the adenovirus (A) genes. This plasmid, Adenovirus type 2 DNA was digested

TABLE 7 (CONT.)

With reference to Tables 2 and 3, the deduced amino acid sequence shown below the nucleotide sequences is numbered beginning with 1 for the first amino acid of the mature protein. The putative leader peptide is indicated by all caps for the amino acid designations. Cysteine residues in the mature protein are additionally indicated by SH and potential N-linked glycosylation sites by an asterisk. The amino acids which are underlined indicate those residues identified by N-terminal protein sequencing or by sequencing tryptic fragments of EPO as described in Example 1. Partial underlining indicates residues in the amino acid

with Hind III and the B fragment was isolated by gel electrophoresis. This fragment was inserted into pBR322 which had previously been digested with Hind III. After transformation of E. coli to ampicillin resistance, recombinants were screened for insertion of the Hind III B fragment and the inserted orientation was determined by restriction enzyme digestion. pBR322 - Ad Hind III B contains the adenovirus type 2 Hind III B fragment in the orientation depicted in Fig. 5B.

As illustrated in Fig. 5B, the VA genes are conveniently obtained from plasmid pBR322 - Ad Hind III B by digestion with Hpa I, adding EcoRI linkers and digestion with EcoRI, followed by recovery of the 1.4 kb fragment. The fragment having EcoRI sticky ends is then ligated into the EcoRI site of PTI, previously digested with EcoRI. After transforming E. coli HB101 and selecting for tetracycline resistance, colonies were screened by filter hybridization to DNA specific for the VA genes. DNA was prepared from positively hybridizing clones and characterized by restriction endonuclease digestion. The resulting plasmid is designated p91023.

As illustrated in Fig. 5C, the two EcoRI sites in p91023 were removed by cutting p91023 to completion with EcoRI, generating two DNA fragments, one about 7 kb and the other about 1.3 kb. The latter fragment contained the VA genes. The ends of both fragments were filled in using the Klenow fragment of polI and the two fragments were then ligated together. A plasmid p91023(A), containing the VA genes and similar to p91023, but deleted for the two EcoRI sites, was identified by Giurstein-Huggness screening with the VA gene fragment, and by conventional restriction site analysis.

The single PstI site in p91023(A) was removed and replaced with an EcoRI site. p91023(A) was cut to completion with PstI and treated with the Klenow fragment of polI to generate flush ends. EcoRI linkers were ligated to the blunted PstI site of p91023(A). The linear p91023(A), with EcoRI linkers attached at the blunted PstI site was separated from unligated linkers and digested to completion with EcoRI, and religated. A plasmid, p91023(B), as depicted in Figure 5C was recovered, and identified as having a structure similar to p91023(A), but with an EcoRI site in place of the former PstI site. Plasmid p91023(B) has been deposited to ATCC 39754.

Production of immunologically active EPO was quantified by a radioimmunoassay as described by Sherwood and Goldwasser (55). The antibody was provided by Dr. Judith Sherwood. The radiolabelled tracer was prepared from the homogeneous EPO described in Example 1. The sensitivity of the assay is approximately 1 ng/ml. The results are shown below in Table 8.

Example 6:

The cDNA clones (lambda-EPOFL6 and lambda-EPOFL13; Example 3) were inserted into the plasmid p91023(B) forming PTFL6 and PTFL13, respectively. 8 ug of each of the purified DNA's was then used to transfect  $5 \times 10^6$  COS cells using the DEAE-dextran method (Intra). After 12 hrs, the cells were washed and treated with Chloroquin (0.1mm) for 2 hrs., washed again, and exposed to 10 ml media containing 10% fetal calf serum for 24 hrs. The media was changed to 4 ml serum free media and harvested 48 hrs. later.

Production of immunologically active EPO was quantified by a radioimmunoassay as described by Sherwood and Goldwasser (55). The antibody was provided by Dr. Judith Sherwood. The radiolabelled tracer was prepared from the homogeneous EPO described in Example 1. The sensitivity of the assay is approximately 1 ng/ml. The results are shown below in Table 8.

TABLE 8

VECTOR	LEVEL OF EPO RELEASED INTO THE MEDIA (ng/ml)
PTFL13	350
PTFL6	31

PTFL13 has been deposited and is available from the American Type Culture Collection, Rockville, Maryland under Accession No. ATCC 39890.

Example 7

EPO cDNA (lambda-EPOFL13) was inserted into the p91023(B) vector and was transfected into COS-1 cells and harvested as described above (Example 6) except that the chloroquin treatment was omitted. *In vitro* biologically active EPO was measured using either a colony forming assay with mouse fetal liver

cells as a source of CFU-E, or a 3H-thymidine uptake assay using spleen cells from phenyhydrazine infected mice. The sensitivities of these assays are approximately 25 mU/ml. *In vivo* biologically active EPO was measured using either the hypoxic mouse or starved rat method. The sensitivity of these assays is approximately 100 mU/ml. No activity was detected in either assay from mock condition media. The results of EPO expressed by clone EPOFL13 are shown below in Table 9 wherein the activities reported are expressed in units/ml, using a commercial, quantified EPO (Troyo, Inc.) as a standard.

TABLE 9

EPO Excreted from COS Cells Transfected with Type EPO cDNA	
Assay	Activity
RIA	100 ng/ml
CFU-E	2.05 U/ml
3H-Thy	3.1 1.8 U/ml
hypoxic mouse	1 U/ml
starved rat	2 U/ml

Example 8: SDS Polyacrylamide Gel Analysis of EPO from COS Cells

180 ng of EPO released into the media of COS cells transfected with EPO (lambda-EPOFL13) cDNA in the vector p91023(B) (supra) was electrophoresed on a 10% SDS Laemmli polyacrylamide gel and electrotransferred to nitrocellulose paper (Towbin et al, Proc. Natl. Acad. Sci. USA 76:4350 (1979)). The filter was probed with anti-EPO antibody as described in Table 8, Western blot, and reprobed with  $^{125}$ I-lactoperoxidase protein. The filter was autoradiographed for two days. Native homogeneous EPO was described in Example 1, either before (lane B) or after iodination (lane C) were electrophoresed (see Figure 6). Markers used included  $^{35}$ S methionine labelled, serum albumin (68,000 d) and ovalbumin (45,000 d).

Example 9: Construction of RKT-4

The Bam HI-PvuII fragment from the plasmid PSV2DHFR (Subramani et al, Mol. Cell. Biol. 1:383-384 (1981)) containing the SV40 early region promoter adjacent to the mouse dihydrofolate reductase (DHFR) gene, an SV40 enhancer, the small t antigen intron, and the SV40 polyadenylation sequence was isolated (fragment A). The remaining fragments were obtained from the vector p91023(A) (supra) as follows: p91023(A) was digested with Pst I at the single Pst I site near to the adenovirus promoter to linearize the plasmid and either ligated to synthetic Pst I EcoRI converters and recircularized (creating the sites Pst I - EcoRI - Pst I at the original Pst I site; 91023(B)) or treated with the large fragment of DNA polymerase I to destroy the Pst I sites and ligated to a synthetic EcoRI linker and recircularized (creating an EcoRI site at the original Pst I site; 91023(B)). Each of the two resulting plasmids 91023(B) and 91023(B) were digested with Xba I and EcoRI to produce two fragments (F and G). By joining fragment F from p91023(B) and fragment G from p91023(B), and fragment G from p91023(B) and fragment F from p91023(B) two new plasmids were created which contained either an EcoRI - Pst I site or a Pst I - EcoRI site at the original Pst I site. The plasmid containing the Pst I - EcoRI site where the Pst I site is closest to the adenovirus major late promoter was termed p91023(C).

The vector p91023(C) was digested with Xba I to completion and the resulting linearized DNA with sticky ends was blunted by an end filling reaction with the large fragment of E. coli DNA polymerase I. To this DNA was ligated a 340 bp Hind III EcoRI fragment containing the SV40 enhancer prepared as follows:

The Hind III - Pvu II fragment from SV40 which contains the SV40 origin or replication and the enhancer was inserted into the plasmid c lac (Little et al, Mol. Biol. Med. 1:473-488 (1983)). The c lac vector was prepared by digesting c lac DNA with BamHI, filling in the sticky end with DNA polymerase I and digesting the DNA with Hind III. The resulting plasmid (c SV40lac) regenerated the

BamHI site by ligation to the Pvu II blunt end. The EcoRI - Hind III fragment was prepared from c SVHPlac and ligated to the EcoRI - Hind III fragment of pSV10d (Mellon et al., supra ) which contained the plasmid origin of replication and the resulting plasmid pSVHPlac was selected. The 340 bp EcoRI - Hind III fragment of pSVHPlac containing the SV40 origin promoter was then prepared, blunted at both ends with the large fragment of DNA polymerase I, and ligated to the XbaI digested, blunted pS1023(c) vector described above. The resulting plasmid (p91023(C)X) had blunt ends EcoRI/Hind III blunt SV40 origin plus enhancer) in which the orientation of the VA gene was such that the BamHI site within that fragment was nearest to the VA gene was termed pES105. The plasmid pES105 was digested with Bam HI and PvuII and also with PvuI alone and the BamHI +PvuI fragment containing the adenovirus major late promoter (fragment B) and the PvuI fragment containing during resistance genes (teracycline resistance) and other sequences (fragment C) were isolated. Fragments A, B and C were ligated and the resulting plasmid shown in Figure 7 was isolated and termed RK1-4. Plasmid RK1-4 has been deposited with the American Type Culture Collection, Rockville, Maryland, where it is available under Accession Number ATCC 38940.

Example 10: Expression of EPO in CHO cells-Method I

DNA (20  $\mu$ g) from the plasmid pPTFL13 described above (Example 6) was digested with the restriction endonuclease Cia I to linearize the plasmid and was ligated to Cia I-digested DNA from the plasmid pJdD26SVPA 1 (2  $\mu$ g) which contains an intact dihydrofolate reductase (DHFR) gene driven by an adenovirus major late promoter (Kaufman and Sharp, Mol. and Cell Biol. 2:1304-1319 (1982)). This ligated DNA was used to transfect DHFR-negative CHO cells (DJKX-BII, Chasin LA, and Utalub G. (1980) PNAS 77: 4216-4220) and following growth for two days, cells which incorporated at least one DHFR gene were selected in alpha media lacking nucleotides and supplemented with 10% dialyzed fetal bovine serum. Following growth for two weeks in selective media, colonies were removed from the original plates, pooled into groups of 10-100 colonies per pool, replated and grown to confluence in alpha media lacking nucleotides. The supernatant media from the pools grown prior to methotrexate selection were assayed for EPO by RIA. Pools which showed positive EPO production were grown in the presence of methotrexate (0.02  $\mu$ M) and then subcloned and reassayed. EPO Cia 4.402-7, a single subclone from the EPO Cia 4.402 pool, releases 480 ng/ml EPO into media containing 0.02  $\mu$ M MTX (Table 10). EPO Cia 4.402-7 is the cell line of choice for EPO production and has been deposited with the American Type Culture Collection as Accession Number ATCC CRL 8895. Currently, this clone is being subjected to stepwise selection in increasing concentrations of MTX, and will presumably yield cells which produce even higher levels of EPO.

For pools which were negative by RIA, methotrexate resistant colonies obtained from the counterpart cultures which were grown in the presence of methotrexate (0.02  $\mu$ M) were again reassayed in pools for EPO by RIA. Those cultures which were not positive were subcloned and subjected to growth in further increasing concentrations of methotrexate.

Stepwise methotrexate (MTX) selection was achieved by repeated cycles of culturing the cells in the presence of increasing concentrations of methotrexate and selecting for survivors. At each round EPO was measured in the culture supernatant by RIA and *In vitro* biological activity. The levels of methotrexate used in each stepwise amplification were 0.02  $\mu$ M, 0.1  $\mu$ M, and 5  $\mu$ M. As shown in Table 10 after 1 round of selection in 0.02  $\mu$ M MTX significant levels of EPO were being released into the culture media.

TABLE 10

Sample	Assay	Level of EPO Released into the Media	
		Alpha medium harvest	0.02 $\mu$ M methotrexate in alpha medium harvest
4.4 Pool	RIA	17 ng/ml	50 ng/ml
4.4 Single Colony Clone (102-7)	RIA	-	480 ng/ml

Example 11: Expression of EPO in CHO cells - Method II

DNA from the clone lambda HEP0113 was digested with EcoRI and the small RI fragment containing the EPO gene was subcloned into the EcoRI site of the plasmid RK1-4. (See Example 10). This DNA (RKFL13) was then used to transfect the DHFR-negative CHO cells directly (without digestion) and the selection and amplification was carried out as described in Example 10 above.

The RKFL13 DNA was also inserted into CHO cells by protoplast fusion and microinjection. Plasmid RKFL13 has been deposited and is available from the American Type Culture Collection, Rockville, Maryland under Accession No. ATCC 38989.

TABLE 11

Sample	Assay	Level of EPO Released into the Media	
		alpha medium harvest	0.02 $\mu$ M methotrexate in alpha medium harvest
Colony Pool A	RIA	3 ng/ml	42 ng/ml (pool)
	3H-Thy	-	150 ng/ml (clone) 1.5 $\mu$ M
Single Colony clone(102C-2)	RIA	-	-
Microinjected pool (DEPO-1)	RIA	60 ng/ml	180 ng/ml
	3H-Thy	1.8 $\mu$ M	-

The preferred single colony clone has been deposited and is available from the American Type Culture Collection, Rockville, Maryland under Accession Number ATCC CRL8895.

Example 12: Expression of EPO Genomic Clone in COS-1 Cells

The vector used for expression of the EPO genomic clone is pSV0d (Mellon et al., supra ). DNA from pSV0d was digested to completion with Hind III and blunted with the large fragment of DNA polymerase I. The EPO genomic clone lambda+HEP03 was digested to completion with Eco RI and Hind III and the 4.0 kb fragment containing the EPO gene was isolated and blunted as above. The nucleotide sequence of this fragment from the Hind III site to a region just beyond the polyadenylation signal is shown in Figure 4 and Table 4. The EPO gene fragment was inserted into the pSV0d plasmid fragment and correctly constructed recombinants in both orientations were isolated and verified. The plasmid C22-1 has the EPO gene in orientation "a" (i.e. with the 5' end of EPO nearest to the SV40 origin) and the plasmid C21-3 is in the opposite orientation "b" (i.e. with the 5' end of EPO nearest to the SV40 origin).

The plasmids C21-3 and C22-1 were transfected into COS-1 cells as described in Example 7 and media was harvested and assayed for immunologically reactive EPO. Approximately 31 ng/ml of EPO was detected in the culture supernatant from C22-1 and 18-31 ng/ml from C21-3.

Example 13: Expression In C127 and in 3T3 Cells Construction of pBPEPO

A plasmid containing the EPO cDNA sequence under the transcriptional control of a mouse metallothionein promoter and linked to the complete bovine papilloma virus DNA was prepared as follows:

The plasmid SP95 was purchased from Promega Bioteck. This plasmid was digested to completion with EcoRI and the 1340 bp EcoRI fragment from lambda-EPOFL13 was inserted by DNA ligase. A resulting plasmid in which the 5' end of the EPO gene was nearest to the SP6 promoter (as determined by *Bgl* I and *Hind* III digestion) was termed pEPO95F. In this orientation, the BamHI site in the PSP605 polylinker is directly adjacent to the 5' end of the EPO gene.

pMMTneo BPV

10 The plasmid pdBPV-mmTneo (342-12) (Law et al., Mol. and Cell Biol. 3:2210-2115 (1983)), illustrated in Figure 8, was digested to completion with BamHI to produce two fragments: a large fragment ~8kb in length containing the BPV genome and a smaller fragment, ~6.5 kb in length, containing the pML2 origin of replication and ampicillin resistance gene, the metallothionein promoter, the tetracycline resistance gene, and the SV40 polyadenylation signal. The digested DNA was recircularized by DNA ligase and plasmids which contained only the 6.8 kb fragment were identified by *Eco* RI and BamHI restrictions endonuclease digestion. One such plasmid was termed pMMTneo BPV.

pEPO15a

20 pMMTneo BPV was digested to completion with *Bgl* II. pEPO46f was digested to completion with BamHI and *Bgl* II and the approximately 700 bp fragment containing the entire EPO coding region was prepared by gel isolation. The *Bgl* II digested pMMTneo BPV and the 700 bp BamHI/*Bgl* II EPO fragment were ligated and resulting plasmids containing the EPO cDNA were identified by colony hybridization with an oligonucleotide probe which is specific for the EPO gene. Of the plasmids which were positive by hybridization analysis, one (pEPO15a) which had the EPO cDNA in the orientation such that the 5' end of the EPO cDNA was nearest the metallothionein promoter was identified by digestion with *Eco* RI and *Kpn* I.

pBPV-EPO

25 The plasmid pEPO15A was digested to completion with BamHI to linearize the plasmid. The plasmid pdBPV-mmTneo (342-12) was also digested to completion with BamHI to produce two fragments of 6.5 and 8.8. The 8.8 kb fragment which contained the entire Bowline Papilloma Virus genome, was gel isolated. The EPO15a/BamHI and the 8.8 kb BamHI fragment were ligated together and a plasmid (pBPV-EPO) which contained the BPV fragment were identified by colony hybridization using an oligonucleotide probe dP-CCACACCGGATACACA-OH which is specific for the BPV genome. Digestion of pBPV-EPO DNA with *Hind* III indicated that the direction of transcription of the BPV genome was the same as the direction of transcription of the metallothionein promoter (as in pdBPV-mmTneo(342-12) see Figure 8). The plasmid pBPV-mmTneo (342-12) is available from the American Type Culture Collection, Rockville, Maryland under Accession No. ATCC 37224.

45 Expression

The following methods were used to express EPO.

50 Method I

DNA pBPV-EPO was prepared and approximately 25 ug was used to transfect ~1x 10<sup>6</sup> C127 (Lowy et al., J. of Virol. 28:291-98 (1978)) CHO cells using standard calcium phosphate precipitation techniques (Graham et al., Virology, 52:458-467 (1973)). Five hrs. after transfection media was removed, the cells were trypsinized, washed, and fresh media containing 10% fetal bovine serum was added. Forty-eight hrs. later, the cells were trypsinized and split at a ratio of 1:10 in DMEM medium containing 500 ug/ml G418 (Southern et al., Mol. Appl. Genet. 1:327-41 (1982)) and the cells were incubated for two-three weeks. G418 resistant colonies were then isolated individually into microtiter wells and grown

until sub-confluent in the presence of G418. The cells were then washed, fresh media containing 10% fetal bovine serum was added and the media was harvested 24 hours later. The conditioned media was tested and shown to be positive for EPO by radioimmunoassay and by *in vitro* biological assay.

5 Method II

50 C127 or 3T3 cells were cotransfected with 25ug of pBPV-EPO and 2ug of pSV2neo (Southern et al., supra.) as described in Method I. This is approximately at 10-fold molar excess of the pBPV-EPO. Following transfection, the procedure is the same as in Method I.

Method III

55 C127 cells were transfected with 30 ug of pBPV-EPO as described in Method I. Following transfection and splitting (1:10), fresh media was exchanged every three days. After approximately 2 weeks, foci of BPV transformed cells were apparent. Individual foci were picked separately into 1 cm wells of a microtiter plate, grown to a sub-confluent monolayer and assayed for EPO activity or antigenity in the conditioned media.

60 Example 14: Expression in Insect Cells Construction of pIVEV EPOFL13

The plasmid vector pIVEV has been deposited and is available from the American Type Culture Collection, Rockville, Maryland under Accession No. ATCC 38991. The vector was modified as follows:

55 pIVEV

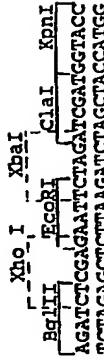
50 pIVEV was digested with *Eco* RI to linearize the plasmid, blunted using the large fragment of DNA polymerase I and a single *Not* I linker GGCGGCCGCC CCACCGAGGG was inserted by blunt end ligation. The resultant plasmid is termed pIVEV1.

55 pIVEV1

50 pIVEV was digested with *Sma* I to linearize the plasmid and a single *Sfi* I linker GGACGCCAGGGGCC CGCGGGATCCCCGGG was inserted by blunt end ligation. The resultant plasmid was termed pIVEVSI.

60 pIVEVSIBgk

45 The plasmid pIVEVSI was digested with *Kpn* I to linearize the plasmid and approximately 0 to 100 bp were removed from each end by digestion with the double-stranded exonuclease *Bal* 31. Any resulting ends which were not perfectly blunt were blunted using the large fragment of DNA polymerase I and the polylinker



polylinker is oriented such that the *Bgl*I site within the polylinker is nearest to the polyhedron gene promoter is termed pIVEVSBgkP. A plasmid in which the *Kpn*I site within the polylinker is nearest to the polyhedron gene promoter is termed pIVEVSKpBgk. The number of base pairs which were deleted between the original *Kpn*I site in pIVEVSI and the polyhedron promoter was not determined. The pIVEVSBgkP has been deposited with and is available from the American Type Culture Collection, Rockville, Maryland under Accession No. ATCC 38888.

#### pIVEVSBgkN1

10 pIVEVSI BgkPNI was digested to completion with *Kpn*I and *Pst*I to produce two fragments. The larger fragment, which contained the plasmid origin of replication and the 3' end of the polyhedron gene was prepared by gel isolation (fragment A). pIVEVSBgkP was digested to completion with *Pst*I and *Kpn*I to produce two fragments and the smaller fragment, which contained the polyhedron gene promoter and the polylinker was prepared by gel isolations (fragment B). Fragment A and B were then joined by DNA ligase to form the new plasmid pIVEVSBgkN1 which contains a partially deleted polyhedron gene into which a polylinker has been inserted and also contains a *Nde*I site (replacing the destroyed *Eco*RI site) and a *Sph*I site which flank the polyhedron gene region.

#### pIVEPO

20 pIVEPO was digested to completion with *Eco*RI to linearize the plasmid and the 1340 bp *Eco*RI fragment from lambda-HEPOFL13 was inserted. Plasmids containing the EPO gene in the orientation such that the 5' end of the EPO gene is nearest the polyhedron promoter and the 3' end of the polyhedron gene were identified by digestion with *Bgl*I. One of these plasmids in the orientation described above was designated pIVEPO.

#### Example 15: Purification of EPO

25 Large amounts of the pIVEPO plasmid were made by transforming the *E. coli* strain JM101-1gl. The plasmid DNA was isolated by cleared lysate technique (Maniatis and Fritsch, *Cold Spring Harbor Manual*) and further purified by CsCl centrifugation. Wild-type *Autographa californica* polyhedrosis virus (AcNPV) strain L-1 DNA was prepared by phenol extraction of virus particles and subsequent CsCl purification of the viral DNA.

30 These two DNAs were then cotransfected into *Spodoptera frugiperda* cells (pLB-Sf-2) (Naughton et al., *Vitro* Vol. 6, pp. 213-17, 1987) using the calcium phosphate transfection procedure (Potter and Miller, 1987). For each plate of cells being cotransfected, 1ug of wild-type AcNPV DNA and 10 ug of pIVEPO were used. The plates were incubated at 27°C for 5 days. The supernatant was then harvested and EPO expression in the supernatant was confirmed by radioimmunoassay and by in vitro biological assay.

#### Example 16: Purification of EPO

35 COS-cell conditioned media (121) with EPO concentrations up to 200ug/ml was concentrated to 800ml! using 10,000 molecular weight cutoff ultrafiltration membranes, such as a Millipore Pallcon fitted with 5 sq. ft. of membrane. Assays were performed by RIA as described in Example 6. The retentate from the ultrafiltration was dialyzed against 4ml of 10mM sodium phosphate buffered at pH7.0. The concentrated and dialyzed conditioned media contained 2.5mg of EPO in 380mg of total protein. The EPO solution was further concentrated to 180ml and the precipitated proteins were removed by centrifugation at 110,000 xg for 30 minutes.

40 The supernatant which contained EPO (2.0mg) was adjusted to pH5.5 with 50% acetic acid, allowed to stir at 4°C for 30 minutes and the precipitate removed by centrifugation at 13,000 xg for 30 minutes.

45 Carboxymethyl Sepharose Chromatography

#### Reverse phase-HPLC

10 The concentrated fractions from CM-Sepharose containing the EPO was further purified by reverse phase-HPLC using Vydac C-4 column. The EPO was applied onto the column equilibrated in 10% solvent B (Solvent A was 0.1% CF<sub>3</sub>CO<sub>2</sub>H in water; solvent B was 0.1% CF<sub>3</sub>CO<sub>2</sub>H in CF<sub>3</sub>CO<sub>2</sub>N) at flow rate of 1ml/min. The column was washed with 10% B for 10 minutes and the EPO was eluted with linear gradient of B (10-70% in 60 minutes). The fractions containing EPO were pooled (~40ug of EPO in 120ug of total proteins) 15 and lyophilized. The lyophilized EPO was reconstituted in 0.1M Tris-HCl at pH7.5 containing 0.15M NaCl and rechromatographed on the reverse phase HPLC. The fractions containing the EPO were pooled and analyzed by SDS-polyacrylamide (10%) gel electrophoresis (Laemmli, U.K., Nature). The pooled fractions of EPO contained 15.5ug of EPO in 25ug of total protein.

20 The invention has been described in detail, including the preferred embodiments thereof. It will, however, be appreciated that those skilled artisans may make modifications and improvements upon consideration of the specification and drawings set forth herein, without departing from the spirit and scope of this invention as set forth in the appended claims.

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## Claims

60 1. Recombinant DNA plasmid vector containing cDNA encoding human EPO of clone lambda HEPFL13 (ATCC 40153).

2. A mammalian cell transformed with the transfer vector of claim 1.

3. The cell of claim 2, wherein said mammalian cell is a 3T3, C127 or CHO cell.

45 4. A mammalian cell containing a plasmid which contains the entire bovine papilloma virus DNA and the cDNA sequences of Table 3 coding for human EPO.

5. The cell of claim 4, wherein said cell is a C127 or 3T3 cell.

6. The cell of claim 5, wherein said EPO DNA is under transcriptional control of a mouse metallothionein

promoter.

7. The cell of claim 5, wherein said cell contains a plasmid comprising DNA from pdBPV-NMTneo (342-12) (ATCC 37224).

8. Recombinant human erythropoietin characterized by the presence of O-linked glycosylation, obtainable by the steps of

(a) culturing in a suitable medium CHO cells containing a DNA sequence encoding human erythropoietin DNA sequence operatively linked to an expression control sequence and recovering and separating the EPO from the cells and the medium.

9. Recombinant human erythropoietin according to claim 8, characterized by a glycosylation pattern comprising fucose.

10. Recombinant human erythropoietin according to claim 9, characterized by a glycosylation pattern comprising relative molar levels of hexoses to N-acetylgalactosamine (Nacgic) of 1.4 : 1, specifically galactose : Nacgic = 0.9 : 1 and mannose : Nacgic = 0.5 : 1.

11. Recombinant human erythropoietin according to claim 9 or 10, characterized by the presence of N-acetylgalactosamine.

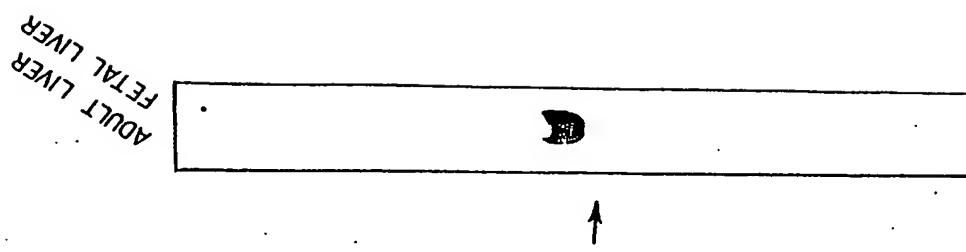


FIG. 1

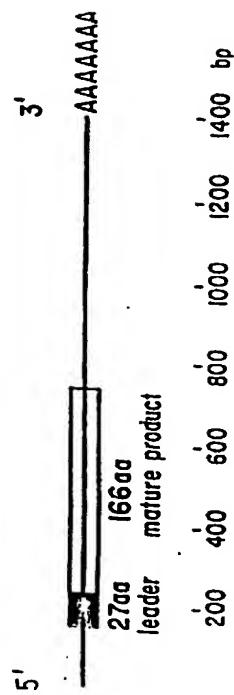
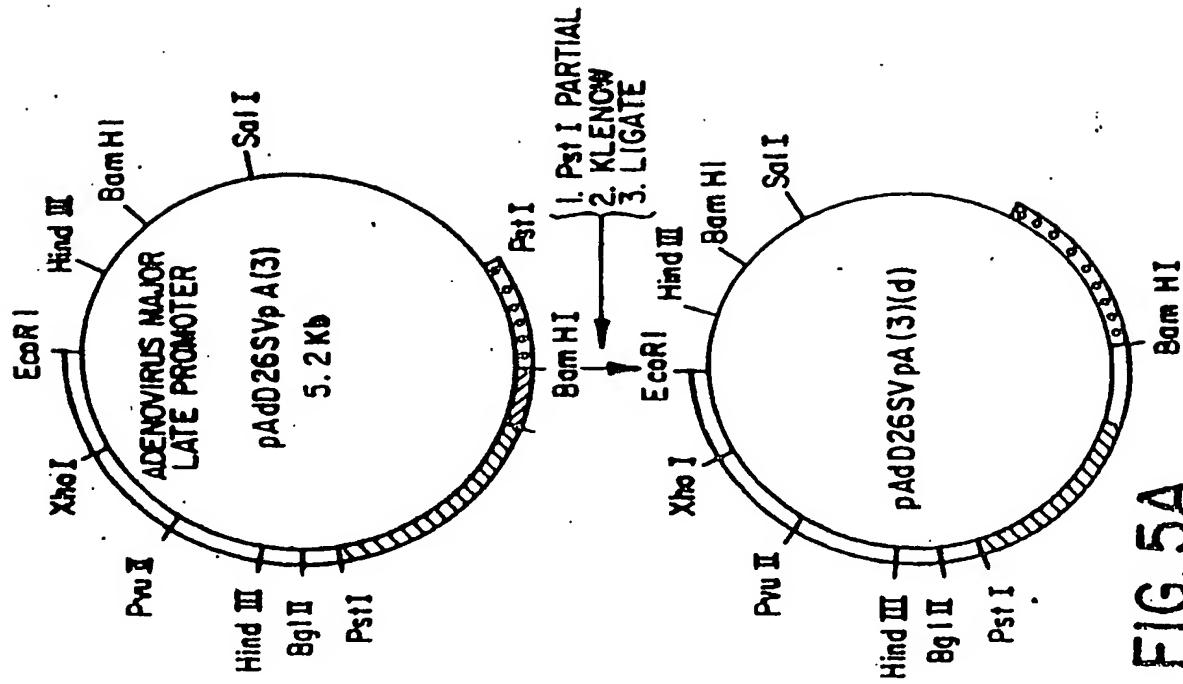
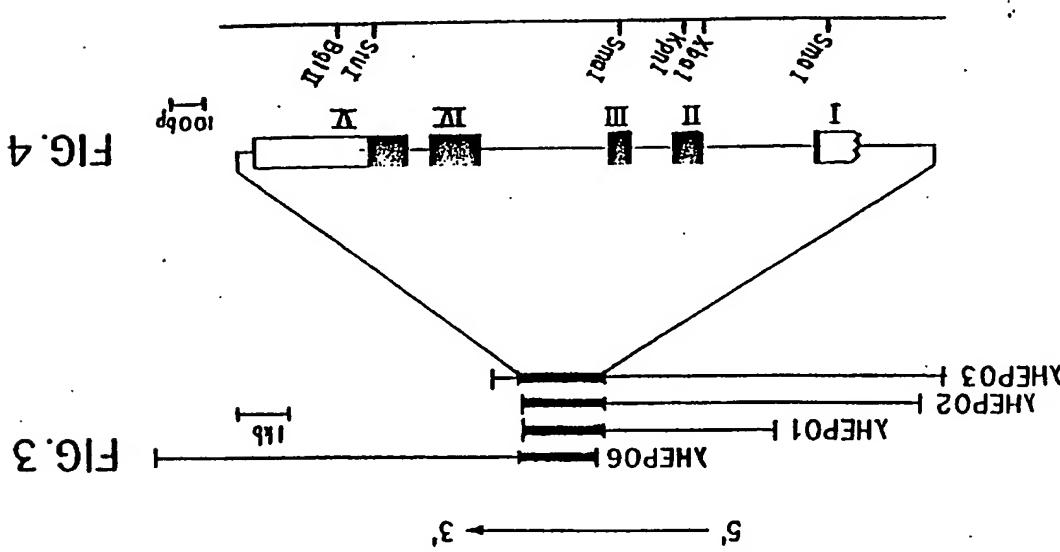


FIG. 2

**FIG. 5A**

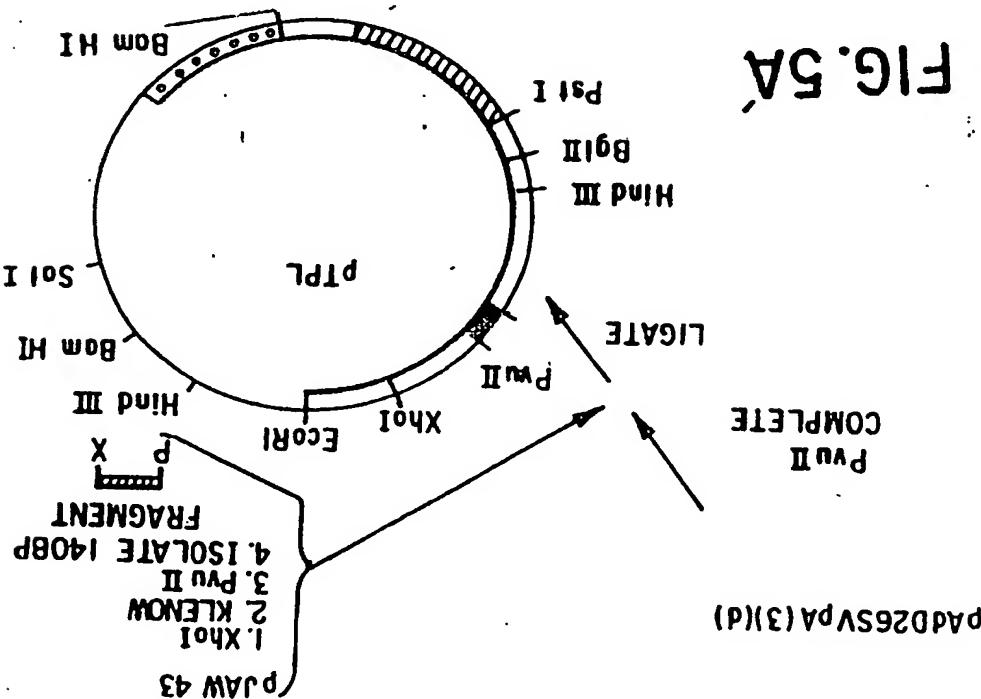
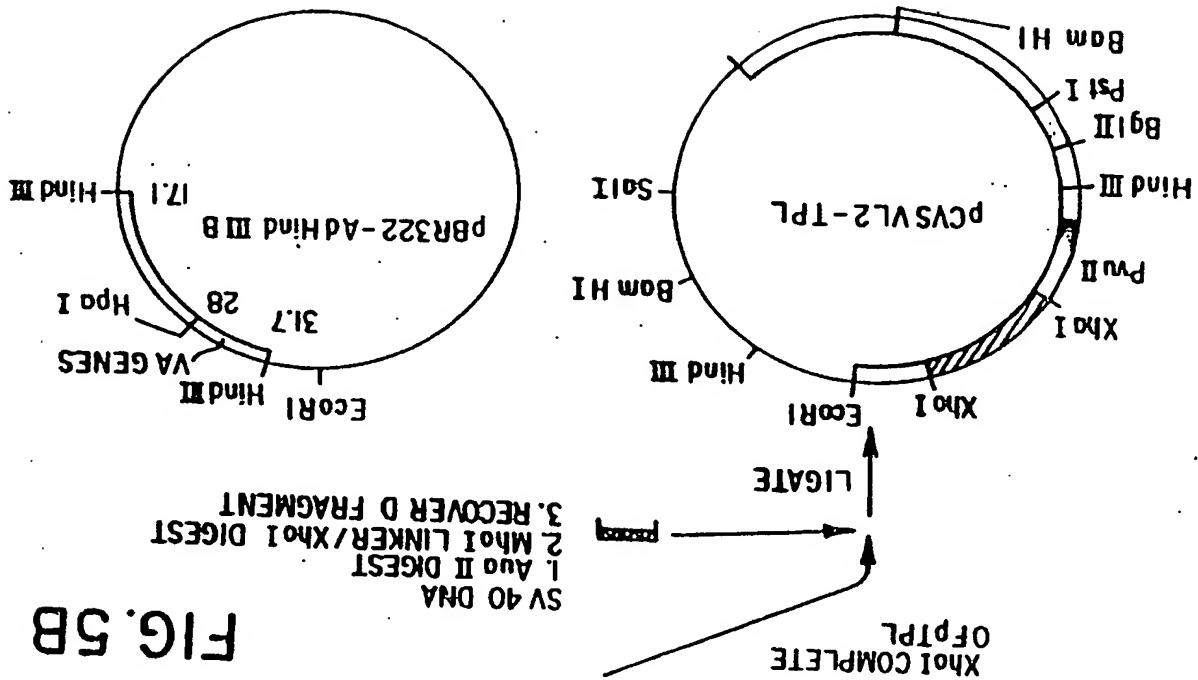


FIG. 5C

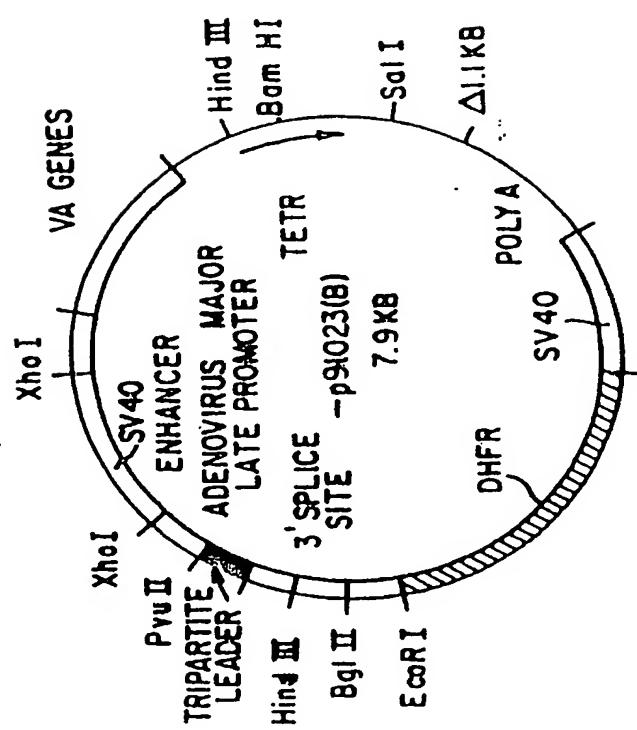
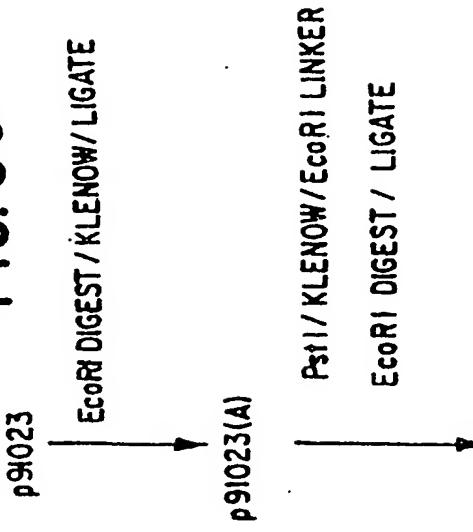
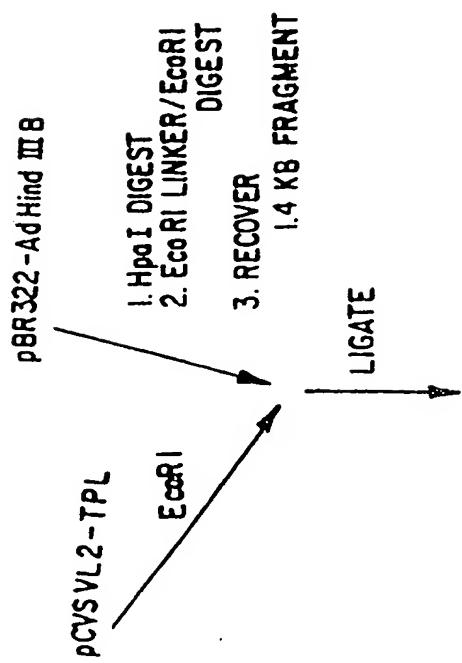
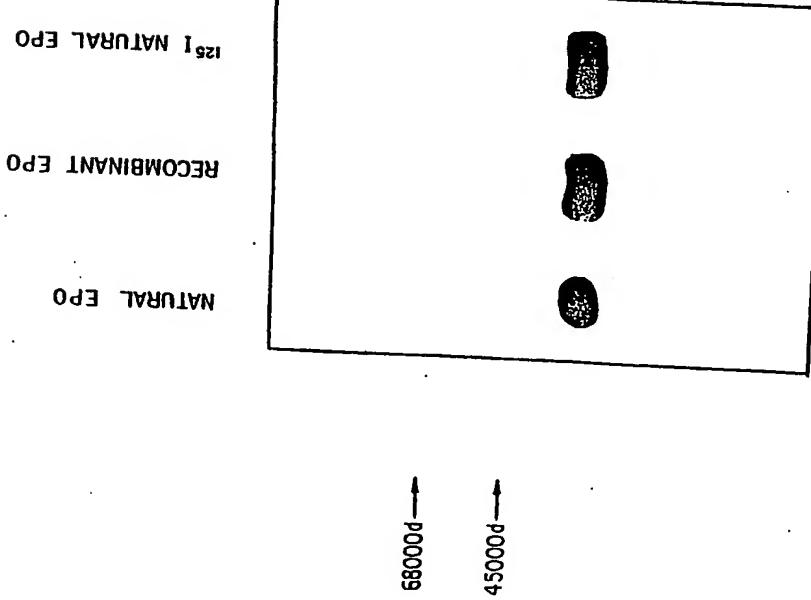
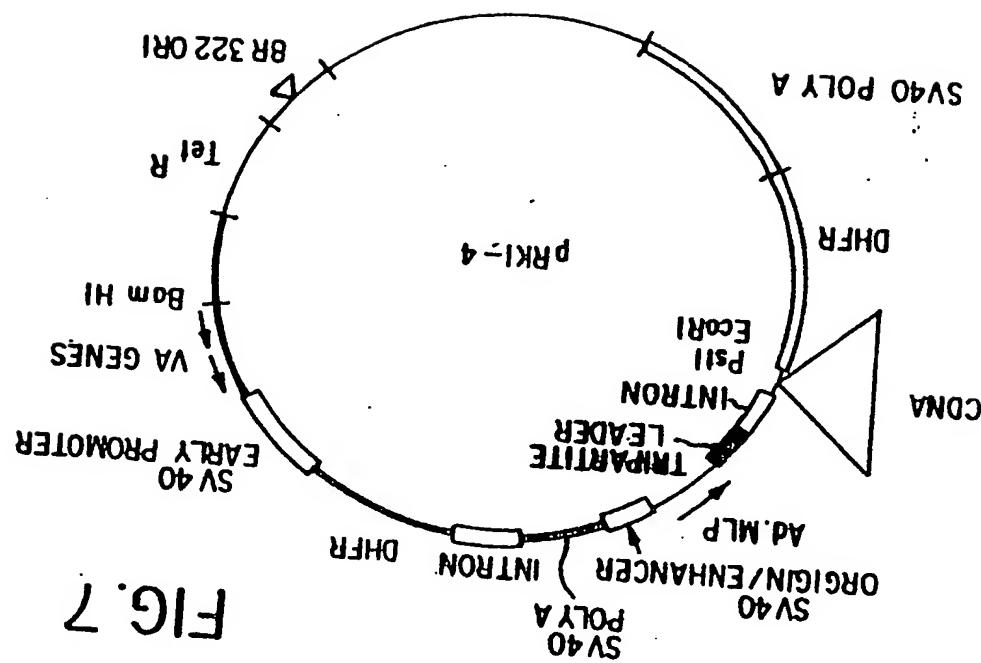


FIG. 5B



DOCUMENTS CONSIDERED TO BE RELEVANT		Element to cite	CLASSIFICATION OF THE APPLICATION (as Cls)	
Category	Character of document, where appropriate, of relevant passages		C 12 N 15/16	C 12 P 21/02
X	PROC. NATL. ACAD. SCI. USA, vol. 81, May 1984, pages 2708-2712; S. LEE-HUANG: "Cloning and expression of human erythropoietin cDNA in <i>Escherichia coli</i> " * Whole document *	1,8	C 12 N 5/10	C 07 K 13/00
X	CHEMICAL ABSTRACTS, vol. 87, 1977, page 8 294, abstract no. 129775g, Columbus, Ohio, US; T. MIYAKE et al.: "Purification of human erythropoietin", & J. BIOL. CHEM. 1977, 252(15), 5558-64 * Whole abstract *	1-3,8-11		
P,X	NATURE, vol. 313, 28th February 1985, pages 806-810; K. JACOBS et al.: "Isolation and characterization of genomic and cDNA clones of human erythropoietin" * Whole document *	1,8		
P,X	WO-A-8 503 079 (NEW YORK UNIVERSITY) * Whole document *	1,8	C 12 N 6/1 K	C 07 K
E	GB-A-2 171 304 (CHUGAI SEIYAKU K. K.) * Reference example 2 *	1-3,8-11		
E,L	CHEMICAL ABSTRACTS, vol. 105, no. 19, 10th November 1986, page 203, abstract no. 166280c, Columbus, Ohio, US; & JP-A-86 12 288 (GENETICS INSTITUTE) 20-01-1986 * Reference example 2 *	1-3,8-11		
A	EXP. HEMATOI., vol. 12, 1984, page 357, abstract no. 1; F. K. LIN et al.: "Cloning and expression of monkey and human erythropoietin gene"	1-3,8-11		

The present search report has been drawn up for all claims

Place of search Date of completion of the search Examiner

THE HAGUE 25-10-1990 DESCAMPS J. A.

EXPLANATION OF CODES

X: Particularly relevant if taken alone  
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P: Intermediate document

EP 0411 678 A1 (continued)

